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(57) Abstract

This invention relates to an assay for molecules capable of interacting with cytokine receptor intracellular peptides, and to peptides suitable for use in said assay. The peptides correspond to tyrosine-containing regions of the sequence of an intracellular domain of a cytokine, a hormone receptor or a JAK Protein Tyrosine Kinase, and comprise a tyrosine which is able to be phosphorylated. In phosphorylated form, the peptides are able to bind cytoplasmic transcription factors of the STAT family.

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"PHOSPHOTYROSINE ASSAY AND PEPTIDES FOR USE THEREIN"

This invention relates to an assay for molecules which are able to interact with cytokine receptor intracellular peptides, in particular receptors of the non-protein tyrosine kinase domain type, and to peptides suitable for use in this assay. In a particularly preferred embodiment, the assay relates to detection of molecules which are able to bind to cytokine receptors.

Background and Prior Art

Cellular proliferation and differentiation in multicellular organisms requires precise coordination and regulation. Binding of extracellular signalling molecules, such as cytokines and growth factors, to specific cell surface receptors provides one major mechanism by which this is achieved. These receptors span the cell membrane, and are classified as to whether or not they possess an intrinsic protein tyrosine kinase domain in their intracellular region. The tyrosine kinase domain phosphorylates tyrosine residues in many intracellular proteins, providing an important step in signal transduction. Receptors which have an intrinsic protein tyrosine kinase are known as receptor tyrosine kinases.

With the advent of the Polymerase Chain Reaction (PCR) many novel members of the receptor protein tyrosine kinase family (RTK; growth factor receptors) have been identified via the highly conserved structural elements within their catalytic domains. Even though these receptors share a common cytoplasmic protein tyrosine kinase (PTK) domain, they have distinctive structural elements in their extracellular domains. These structural elements can be used to classify the RTKs into a given subfamily, and demonstrate the structural diversity that exists within the RTK family (Wilks, 1989).

The signals which are received by the transmembrane RTKs are transmitted further downstream, and eventually into the nucleus of the cell, by other signal

transduction mol cules which have SRC Homology 2 (SH2) domains. These SH2 domains are regions of protein sequences (modules) of approximately 100 amino acids which bind independently to phosphotyrosine residues. The binding demonstrates high affinity and amino acid sequence specificity. The direct, sequence-specific interaction of SH2 domains with extracellular signal-induced phosphotyrosine residues has been recognised as a unifying theme in intracellular signal transduction.

The provision of high affinity and specificity in this interaction appears to be the most important factor defining the intracellular outcome of extracellular interactions. For cell-surface RTKs which are members of the protein tyrosine kinase (PTK) family, it is now well documented that recruitment of substrates to the activated receptor is mediated by the phosphorylation of tyrosine residues in the intracellular domain of the receptor. The specific amino acid context in which these phosphotyrosines are embedded defines which "second-wave" substrates are bound to the receptor and become substrates for its PTK activity.

Thus these phosphotyrosine residues form a series of molecular tags to which proteins containing SH2 domains are attracted. Association of the SH2-bearing signal transducers with the activated receptors leads in turn to the activation of the transducers, often due to tyrosine phosphorylation by the RTK. This provides a mechanism whereby a particular receptor can select a specific subset of SH2-containing proteins to be bound, and subsequently modified and activated. Thus all signal transduction processes triggered by the activation of a RTK are coordinated by the SH2 domain-phosphotyrosine nexus.

The JAK family of PTKs (Just Another Kinase or JAnus Kinase) were discovered by polymerase chain reaction-based screening using degenerate oligonucleotide primers based on highly conserved PTK catalytic domain motifs. See for example Australian Patent Application No. 88229/91.

For many receptors that are not themselves PTKs, members of the JAK family of PTKs are employed as rec ptor "β chains", and these molecules then act in much the same way as do the intrinsic PTK domains of the RTK family. While JAK PTKs do not possess obvious SH2 and SH3 domains, they do have a PTK domain (JH1) and a kinase-related domain (JH2), as well as 5 other JH domains.

The cell surface receptors for a large proportion of haemopoietic growth factors (lymphokines), including IL-3 and IL-6, while not themselves members of the PTK family, can be grouped together with the receptors for growth hormone, prolactin, leukaemia inhibitory factor, erythropoietin and ciliary neurotrophic factor, and other hormones and growth factors such as oncostatin M, to form an extended family of cytokine receptors. Members of this family are characterised by the presence of four highly conserved cysteine residues in the extracellular domain, and, except for the growth hormone receptor, the characteristic sequence motif -WSKWS-, usually close to a single transmembrane domain. Although the cytokine receptors have little significant amino acid sequence homology within their intracellular domain, they are clearly an evolutionarily-related set of cell surface receptors. They do not possess any protein kinase-related sequences, but it is well known that interaction of these receptors with their respective ligands triggers a cascade of tyrosine phosphorylation in the target cell. Thus while these receptors are not themselves members of the PTK family, recruitment of PTK activity is an important step in propagation of intracellular signalling pathways downstream of cytokine receptors, and in fact it has been shown that members of the JAK family of PTKs act as downstream transduction factors for these receptors.

These non-PTK cytokine receptors fall into two classes. Type I cytokine receptors either require ligand-induced homodimerization for signal transduction, for example erythropoietin receptor and growth hormone

receptor, or require recruitment of at least two distinct sub-units for effective high-affinity ligand binding and signal transduction. In the latter group, in many cases several different ligand-specific α sub-units are capable of combining with the same β sub-unit. For example, the α sub-units of the receptors for IL-3, IL-5 and GM-CSF all combine with the same β sub-unit.

The Type II cytokine receptors include the receptor for interferon-α and interferon-β (the IFN-α/β receptor) and the interferon-γ receptor. While interferon-α and interferon-β appear to share a common receptor, interferon-γ binds to a separate receptor, and there is little or no sequence homology between the two groups of interferons. However, the cellular response elicited by the two receptor types shares common features, suggesting that they may recruit very similar intracellular signal transduction pathways.

Stimulation of cells by interferon molecules leads to activation of latent cytoplasmic transcription factors which migrate to the nucleus and bind specific recognition sequences located within the promoter regions of various interferon-inducible genes. In the case of IFN-α, the cytoplasmic transcription factor is designated interferon-stimulated gene factor 3α (ISGF-3α), which in turn binds to an interferon-stimulated response element (ISRE), which is required for induction of transcription.

The ISGF-3α complex consists of three polypeptides, formerly designated p113, p91 and p84, but now known respectively as STAT2, STAT1α and STAT1β, where STAT stands for Signal Transducers and Activators of Transcription. The STAT molecules are a family of related transcription factors. So far, 5 genes have been described in the literature, although about 10 are thought to exist.

STAT1 has two alternatively spliced forms, previously designated p91 or STAT91 and p84 or STAT84 respectively. They are now called STAT1α and STAT1β respectively. STAT2 is encoded by a different gene, and

the protein was previously known as p113 or STAT113.

STAT3, STAT4, STAT5 and STAT6 have been identified, and encode proteins that are also involved in an analogous manner to STAT1α, STAT1β and STAT2 in signal transcription from various cytokine receptors.

This subject has recently been reviewed by two of the present inventors (Wilks and Harpur, 1994).

As discussed in that review, a great many growth factors and cytokines utilise common intracellular signalling molecules to generate a nuclear response. Pathways activated by EGF, PDGF, IFN-α, IFN-γ, CSF-1 and IL-6 utilise homologous DNA promoter sequences and common transcription factor components. While the IFN-α receptor utilises STAT2, STAT1α and STAT1β, the receptors for IFN-γ, EGF, PDGF and CSF-1 use STAT1α and other as yet unidentified phosphoproteins. There is also redundancy or overlap of the DNA response elements involved. For example, a number of cytokines cause activation of DNA binding proteins which recognise a single region, designated the IFN-γ response region (GRR), located within the promoter of the high-affinity Fc-γ receptor gene. Thus IL-3, IL-5, IL-10 and GM-CSF all ultimately activate transcription factors which bind to sequences within a single element, further demonstrating the overlap and degeneracy involved in intracellular signal transduction.

The specific phosphotyrosine-containing motifs on cytokine receptors which attract members of the STAT family via their SH_2 domains are at present poorly defined. One exception is the STAT binding site of the IFN-γ receptor. The tyrosine located at amino acid position 440 in the human sequence has been shown to be the most important residue in defining the binding of STAT1α, and therefore in the commitment of the IFN-γ treated cell to respond to this cytokine in the appropriate way.

As a model, a 16-amino acid peptide was synthesized, designated Y440, whose sequence corresponds to part of the sequence of the IFN-γ receptor

(KAPTSFGYDKPHVLVD; SEQ. ID. NO. 1), a biosensor (BIAcore™)-based assay using the interaction of STAT1 from crude cellular extracts with Y440 on a BIAcore™ chip was developed. The assay is simple (it can be used to assay crude cellular extracts, with Y440 on a BIAcore™ chip), reproducible (the signal is strong and easily read), and dependent upon the presence of phosphotyrosine in the target peptide. It has been further demonstrated that the binding is exclusively due to STAT1, and that the binding is both specific and of high affinity. Thus, a robust assay for agonists, antagonists, modulators and mimics of the STAT1/Y440-type interaction has been devised.

Essentially the signal transduction pathway of IFN-γ has been broken down into a series of protein-protein interactions, and the most specific of them has been targeted. This assay therefore provides a means of rational drug design and/or screening for agonists, antagonists, modulators or mimics of the effects of IFN-γ on cell metabolism, including but not limited to screening of natural products.

The same basic mechanism is predicted to operate for all cytokines which act through the JAK kinase-STAT nexus. For example, the corresponding STAT2 binding site on the α chain of the IFN α/β receptor has been identified. This is IFN-α R [Y466p], corresponding to residues 460-474 of the IFN-α receptor (FLRCINYVFFPSLKP; SEQ. ID. NO. 2). An IFN α/β, IL-2, IL-4, IL-5, IL-9, IL-13, IL-15, GM-CSF, growth hormone or prolactin specific drug screen is envisaged along the same lines.

Antagonists of IL-4 and IL-5 are useful as anti-atopy drugs in the treatment of conditions such as asthma and psoriasis. Antagonists of GM-CSF are useful in the treatment of inflammatory conditions such as rheumatoid arthritis. Antagonists of interferon are useful in the treatment of autoimmune diseases such as insulin-dep ndent diabetes mellitus. Agonists of interferon and other immune mediators are useful in the treatment of neoplastic

disease, including leukaemias. Antagonists of haemopoietic growth factors are useful in the treatment of blood dysplasias involving overproduction of blood cells, e.g. erythropoietin antagonists for treatment of overproduction of red cells, and antagonists of GM-CSF or CSF-1 for treatment of overproduction of white cells.

Summary of the Invention

According to a first aspect the invention provides a peptide corresponding to a tyrosine-containing region of the sequence of an intracellular domain of a cytokine or hormone receptor or of a JAK protein tyrosine kinase, wherein said peptide comprises a tyrosine residue which is able to be phosphorylated, and in its phosphorylated form is able to bind a cytoplasmic transcription factor of the STAT family.

It will be clearly understood that the invention is applicable to any cytokine and its cognate STAT molecule. For example some cytokines or hormones preferentially interact with STAT1α, some with STAT1β, and some with STAT2. The skilled person will be able to identify the most appropriate STAT molecule to use with each desired cytokine receptor phosphopeptide, using methods known in the art or described herein.

Preferably the peptide, referred to herein as the receptor peptide, is derived from a receptor for a protein selected from the group consisting of growth hormone, prolactin, leukaemia inhibitory factor, erythropoietin, ciliary neurotrophic factor, interferon-γ, interferon-α, interferon-β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF, CSF-1, erythropoietin, EGF, PDGF and Oncostatin M.

More preferably the peptide is derived from a receptor for a protein selected from the group consisting of growth hormone, prolactin, interferon-γ, interferon-α, interferon-β, IL-2, IL-4, IL-5, GM-CSF and erythropoietin.

In one particularly pr ferr d embodiment, the invention provides a peptide having the sequence KAPTSFGYDKPHVLVD (SEQ. ID. NO. 1), corresponding to residues Lys_{433} to Asp_{448} of the amino acid sequence of the interferon-γ receptor protein, which in its phosphorylated form has the ability to bind STAT1. The peptide and its phosphorylated form are referred to herein as IFN-γ R [Y440] and IFN-γ R [$Y440_p$] receptor.

In a second particularly preferred embodiment, the peptide has the sequence FLRCINYVFFPSLKP (SEQ. ID. NO. 2), corresponding to residues Phe_{460} to Pro_{474} of the interferon-α receptor protein, which in its phosphorylated form has the ability to bind STAT2. This peptide and its phosphorylated form are referred to herein as IFN-α R [Y466] and IFN-α R [$Y466_p$] respectively.

It will be clearly understood that homologues or variants of these peptide sequences are within the scope of the invention, provided that the relevant tyrosine is still present and able to be phosphorylated, and provided that the ability of the phosphorylated peptide to bind a STAT molecule is retained.

The peptides of the invention may be prepared by any convenient method; for example, they may be synthesised chemically, using conventional solid-phase techniques, they may be produced by recombinant DNA methods, or they may be prepared by cleavage of the appropriate receptor protein using appropriate enzyme techniques. The receptor protein may be prepared by any convenient method.

The peptides of the invention are useful in the screening of molecules for their ability to interact with the receptor proteins. For example the peptides of the invention may be used to screen for inhibitors of interferon-γ, interferon-α, interferon-β or of other cytokin s, such as interleukins, especially interleukin-4, interleukin-5 and interleukin-6, GM-CSF, G-CSF, other haemopoietins, including but not r stricted to leukaemia inhibitory factor, and ciliary neurotrophic factor, and

hormones and growth factors, such as growth hormone and EGF.

All cytokines which use the same basic signal transduction mechanisms as those of interferon-γ are suitable for use with the peptides of this invention, especially IFN-γ R [$Y440_p$]. Thus any molecule whose activity is mediated by a STAT molecule or by the JAK-STAT pathway is within the scope of the invention. The person skilled in the art will readily be able to test whether the receptor for a given protein belongs in this class, to identify a suitable phosphorylation site for use in the assay of the invention, and to prepare an appropriate peptide, using methods already known in the art.

The complete amino acid sequences for many cytokine or hormone receptors are already known, or can be determined using routine methods. From the sequence, tyrosine residues susceptible to phosphorylation can readily be identified. Tyrosine(s) which are important to interaction with the cognate STAT molecule can be rapidly identified using routine site-directed mutagenesis. Once the relevant tyrosine residue is identified, conveniently-sized peptides can rapidly be synthesized and tested.

Accordingly, in a second aspect the invention provides a method of screening of molecules for their ability to interact with a receptor peptide, comprising the step of exposing a receptor peptide to a molecule to be screened, and measuring the degree of interaction between the peptide and the molecule.

According to a third aspect, the invention also provides a method of measuring the ability of a molecule to inhibit or promote interaction between a receptor peptide and a molecule known to be able to bind to said receptor, comprising the step of exposing the receptor peptide to a known molecule having the ability to bind to said receptor in the presence of a putative inhibitor or promoter, and measuring the ability of the putative inhibitor to inhibit or promote said binding.

For the purposes of this specification, the term "interact" is to be understood to encompass promoting or inhibiting the activity of the receptor peptide. Thus a molecule which interacts with a receptor peptide may act as an agonist or antagonist of the receptor, may mimic the activity of the receptor, or may modulate its activity directly or indirectly. Moreover, the interaction may take place either during the screening process, or may take place prior to screening. Thus this aspect of the invention includes within its scope detection of interactions which take place *in vitro* or *in vivo*, before preparation of the sample which is actually subjected to the assay, as well as interactions taking place within the sample. It is particularly envisaged that cell or tissue cultures whose medium is to be assayed, or an animal from which a tissue sample or biological fluid is to be assayed, may be pretreated with the molecule to be screened for its ability to modulate the interaction between a receptor peptide with its cognate STAT.

In both the second and third aspects of the invention, the molecules to be screened may be of either synthetic, recombinant or natural origin, and may be of a wide variety of structures.

In both the second and third aspects, the binding may be measured by any convenient means. For example, either the receptor peptide or the molecule to be tested for binding may be labelled with a detectable marker, such as a radioactive label, a fluorescent label, or a marker detectable by way of an enzyme reaction. Many suitable detection systems are known in the art. Thus assay systems which are suitable for use in the methods of the second and third aspects of the invention include, but are not limited to, immunoassays such as enzyme linked immunosorbent assay (ELISA); affinity-type assays such as those using coated microtitre plates, beads or slides, or affinity chromatography; fluorescence-activated cell sorting; biosensor assays; and bioassays.

One particularly rapid and convenient test system uses an optical biosensor, such as the BIAcore™ (Pharmacia Biosensor AB, Uppsala, Sweden), which enables the use of proteins or peptides immobilized to a sensor chip. The biosensor assay is very simple, reproducible and rapid, while having high specificity. The biosensor assay enables a very high throughput of samples, and is amenable to automation, and to use with relatively crude samples, such as biological fluids, or cell or tissue extracts. It is therefore suitable for screening of natural products for their ability to inhibit or promote binding, or for activity as agonists or antagonists of cytokines. It is also suitable for use with biological fluids, for example in clinical assays, or culture media, including medium from recombinant bacterial or cell cultures.

The BIAcore™ assay of the invention permits qualitative or quantitative analysis of the effects of putative modulators, including agonists and antagonists, on the kinetics of the STAT/STAT and STAT/receptor peptide interaction. Potentiation of STAT binding or decrease in affinity for STAT are characteristics which may be desirable in a given potential therapeutic agent.

Detailed Description of the Invention

Abbreviations used herein as are as follows:

BSA	bovine serum albumin
FCS	foetal calf serum
GM-CSF	granulocyte-macrophage colony-stimulating factor
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
IFN	interferon
IL	interleukin
MAb	monoclonal antibody
PTK	protein tyrosine kinase
RTK	receptor tyrosine kinase
RU	response units

STAT signal transducers and activators of transcription.

The invention will now be described by way of reference only to the following non-limiting examples, and to the figures.

Figure 1 shows a sensorgram profile of immunological detection of the phosphorylated tyrosine-containing peptide IFN-γ R [$Y440_p$] immobilized to a BIAcore™ sensor chip, using a phosphotyrosine-specific MAb

_____Δ 1 μg/ml antiphosphotyrosine monoclonal antibody, IFN-γ R [$Y440_p$]-derivatised sensor chip;

_____o 1 μg/ml anti-phosphotyrosine MAb and peptide IFN-γ R [Y440]-derivatised sensor chip;

_____◇ 1 μg/ml anti-STAT1 monoclonal antibody, peptide IFN-γ R [$Y440_p$]-derivatised sensor chip.

Figure 2 shows the relative response of IFN-γ R [$Y440_p$] peptides to crude lysates of HeLa 3 cells.

Figure 3 shows the correlation between the relative response of an IFN-γ R [$Y440_p$]-coupled biosensor chip with the presence of immunoreactive STAT1 in the cytosolic fraction of a HeLa S3 cell extract.

Figure 4 shows the effect of depletion of STAT1 from a nuclear extract of HeLa S3 cells on the response of a biosensor coupled to IFN-γ R [$Y440_p$].

Figure 5 shows a sensorgram demonstrating immunodetection of STAT1 binding to an IFN-γ R [$RY440_p$]-coupled sensor chip.

Figure 6 shows

A: Immunoblot detection of STAT1 in cytosolic extracts of cell lines expressing STAT1, but not in a mutant cell line which does not express this protein;

B: Sensorgrams showing immunodetection of IFN-γ R [$Y440_p$] binding activity of these cell extracts;

C: Comparison between binding of IFN-γ R [$Y440_p$] and IFN-α R [$Y466_p$] by the

cell extracts; and

D: Immunoblot detection of STAT1 in peptide-affi agarose treated samples of cell extracts by anti-STAT1 MAb.

Example 1 Immunological detection of IFN-γ receptor (R) peptides, immobilized on to activated BIAcore™ Sensor chips

The 16 amino acid peptide IFN-γ R [Y440], having the sequence KAPTSFGYDKPHVLVD corresponding to residues Lys_{433}-Asp_{448} within the cytoplasmic domain of the IFN receptor, and its tyrosine-phosphorylated homologue IFN-γ R [$Y440_p$], encompassing the functionally important residue Tyr_{440} of the IFN-γ receptor α chain (Farrar *et al*, 1992; Greenlund *et al*, 1994), were each synthesised by conventional solid-phase Fmoc chemistry, purified by either precipitation and by preparative reversed phase (RP)-HPLC, and their identity confirmed by amino acid analysis, ion spray mass spectrometry (Nash *et al*, 1993) and microbore RP-HPLC with on-line UV spectroscopy.

The application of the BIAcore™ biosensor for the analysis of biomolecular interactions (Fägerstam, 1991; Jönsson *et al*, 1991; Jönsson and Malmqvist, 1992), in particular for the analysis of phosphopeptide/protein interactions, has been described elsewhere (Felder *et al*, 1993; End *et al*, 1992; Panayotou *et al*, 1993) and in application notes issued by Pharmacia. Unless otherwise stated, the running buffer used in the BIAcore™ experiments was 20 mM HEPES, pH 7.4, 150 mM NaCl, 2.8 mM EDTA, 0.005% Tween 20. For peptide derivatisation of BIAcore™ sensor chips (CM5, Pharmacia), carboxyl groups of the carboxymethylated dextran matrix were activated with a 1:1 mixture of N-hydroxysuccinimide (NHS) and 200 mM N-ethyl-N"-(3-diethylaminopropyl)-carbodiimide (EDC), both from Pharmacia, as previously described (Nice *et al*, 1993), and 45 μl of purified peptides (>98% purity by microbore RP-HPLC, ion spray mass spectrometry) at

2 mg/ml in 50 mM HEPES, pH 7.5, containing 0.15 M NaCl were injected on to the matrix at 2 μl/min. Excess reactive NHS groups were blocked with 30 μl 1.0 M ethanolamine at 2.0 μl/min. Following the immobilization the baseline response level had increased by 450 and 400 Relative Response units (RU) for IFN-γ R [Y440] and IFN-γ R [$Y440_p$], respectively, suggesting surface concentrations of 0.45 and 0.4 ng/mm^2.

The phosphotyrosine-specific MAb and the anti-STAT1 MAb were diluted into BIAcore™ running buffer at 1 μg/ml prior to injection. The anti-STAT1 MAb was provided at 0.25 mg/ml in a solution containing 1.0 mg/ml BSA and 50% glycerol as stabilizers. The presence of these additives does not have an effect on the immunoreactivity of the MAb (see Example 5), but is evident in the sensorgram as a substantial refractive index change.

A 35 μl sample containing 1 μg/ml of a phosphotyrosine-specific MAb (Upstate Biotechnology Incorporated, Lake Placid, NY, USA. Cat. No. 05-321) was passed at 5 μl/min over a BIAcore™ sensor chip which had been derivatised with approximately 0.5 ng of IFN-γ receptor-derived peptide [Y440]. Following the sample pulse the chip surface was washed for 750 s with running buffer followed by injection of 30 μl of 20 mM phenylphosphate to desorb phosphopeptide-bound MAb. The sensorgram profile is shown in Figure 1. Injection of sample, end of injection and injection of desorption solution are indicated with ^1, ^2, ^3, respectively. In a concurrent experiment an identical sample was injected over a parallel channel of the same sensor chip derivatised with the corresponding tyrosine-phosphorylated peptide IFN-γ R [$Y440_p$]. Furthermore, a non-relevant MAb (anti-STAT1 MAb, Transduction Laboratories, Lexington, KY, USA. Cat. No. G16920) was injected over the IFN-γ R [$Y440_p$]-derivatised channel to provide a control for the BIAcore™ response in this and a subsequent experiment (Example 5).

Example 2 BIAcore™ responses of immobilized IFN-γ R [Y440] to crude HeLa lysates

Cell lysates were prepared as follows. Hela S3 cells were grown in RPMI +10% FCS under 5% CO_2 in a 3L spinner flask (Bellco Biotechnology) to a density of 5-8x10^5 cells.mL^{-1}. Cells were stimulated for 12-16 hours (priming) or 15 minutes (acute) with 5 ng.mL^{-1} human recombinant IFN-γ (Genentech). Cytoplasmic extracts were prepared after appropriate stimulation as described by Lock *et al* (1991), with the following modifications. Cells were washed in ice-cold PBS, then resuspended in ice- cold hypotonic lysis buffer [5 mM Tris.HCl pH 7.5, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, protease inhibitors (1 mM phenylmethyl sulphonyl fluoride (PMSF) , 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μM pepstatin, 1 mM 1,10-phenanthroline, 5 μM E64) and tyrosine phosphatase inhibitor (0.1 mM sodium orthovanadate) at a density of 1x10^7/ml, and then disrupted by Dounce homogenisation (to 80% disruption). Insoluble material was removed by centrifugation at 1000 g for 4 minutes. The supernatant was then diluted 2x with hypotonic lysis buffer containing 20% sucrose, and clarified by centrifugation at 100,000 g for 30 minutes.

Clarified supernatant was then concentrated 7 to 10-fold in an Amicon Centricon concentrator (Amicon, Cat. No. 4304). Prior to the binding experiments, 2.5 ml aliquots of concentrated cell extracts were buffer exchanged into an assay buffer containing 50 mM HEPES, pH 7.0, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, protease inhibitors (10 μM each of leupeptin and pepstatin, 5 μM E64, 1 mM 1,10-phenanthroline, 1 mM PMSF, 1 mM EDTA) and phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM NaF) using PD10 or NAP5 desalting cartridges (Pharmacia), with approximately 95% efficacy (according to manufacturer's notes).

The freeze-dried phosphopeptide IFN-γ R [$Y440_p$] was dissolved in a volume of 700 μl at 2 mg/ml into DMSO

and incubated with 500 µl of Affi-10 NHS-activat d agarose (BioRad) for 8 hours at room temperature. Non-reacted NHS groups of the affinity resin were blocked by incubation with 1 M ethanolamine-HCl, pH 8.5. The immobilization of peptide was monitored by microbore RP-HPLC (with on-line UV-spectroscopy) of samples taken prior to and following the coupling and blocking steps, and coupling efficiency was estimated from the peak area (absorbance at 214 nm) obtained for the analysed peptide solutions. The coupling efficiency under these conditions was 20%, yielding 280 µg peptide/ml of agarose.

For binding studies, 1.5 ml of 7-fold concentrated HeLa extract was incubated on an end-over-end rotator for 30 minutes at room temperature with 0.5 ml IFN-γ R [$Y440_p$]-coupled agarose. Samples (100 µl) were taken prior to the incubation and after 10, 20 and 30 minutes, and passed over an IFN-γ R [$Y440_p$]-derivatised sensor chip at 5 µl/minute. The BIAcore™ running buffer for these experiments was similar to the assay buffer, but did not contain protease inhibitors and glycerol.

Samples of 10-fold concentrated cytosolic extracts of HeLa cells, or dilutions thereof, were incubated either with free IFN-γ R [$Y440_p$] phosphopeptide or with this peptide immobilized to NHS-activated agarose (Affi-gel 10, BioRad). Aliquots of the former samples and of 100,000 x g supernatants (s/n) aspirated at indicated times off the IFN-γ R [$Y440_p$]-Affi-gel 10, or dilutions thereof, were injected at 5 µl/minute on to BIAcore™ sensor chips, derivatised on parallel channels with IFN-γ R [Y440] or IFN-γ R [$Y440_p$] as described in Example 1. The increase of BIAcore™ responses following injection of the sample plugs (Relative Response, RU) were recorded at a defined time point (after position ^2, as indicated in Example 1) and are shown in Figure 2. After each cycle, any material remaining bound to the surface was removed by injection of a 20 µl-pulse of 10 mM HCl containing 0.5 M NaCl.

Example 3 Correlation of IFN-γ R [Y440$_p$] BIAcore™ response with immunoreactive STAT1 in SE-HPLC fractions of crude cytosolic HeLa extracts

Aliquots (0.5 ml) of 10-fold concentrates of the cytosolic fractions of HeLa cells, which had been cultured for 16 hours with 5 ng/ml IFN-γ and then stimulated with an additional 5 ng/ml IFN-γ 15 minutes prior to harvest, were fractionated at 0.25 ml/minute in assay buffer (without glycerol, protease inhibitors or NaF) on a size exclusion HPLC column which had been calibrated with various proteins of known molecular radius (M_r).

Size exclusion-HPLC was performed at 0.25 ml/minute on a Superose-12 column (10x300 mm, Pharmacia) using a Waters Model 626 Protein purification system. Protease inhibitors and NaF were omitted from the HPLC running buffer, since they resulted in critically increased backpressure during the separation. Calibration of the size exclusion-HPLC column with a mixture of standard proteins yielded a linear correlation (r=0.9) between the log M_r and retention time for the molecular weight range between 17 and 670 Kd. Column fractions which were not tested immediately were stored at 4°C overnight or at -70°C for extended periods, without loss in binding activity.

Analysis on the biosensor was performed in the modified assay buffer as described in Example 2. The sample rack of the instrument was kept at 14°C during the course of the analysis.

Samples (35 µl) of each eluting 1 minute fraction were passed over parallel channels of the same sensor chip derivatised with IFN-γ R [Y440$_p$] and IFN-γ R [Y440] peptides, respectively. The relative responses (as defined in Example 2) in ach fraction are shown in Figure 3A. No response to the immobilized, non-phosphorylated IFN-γ R peptide [Y440] was seen in any of the fractions. Remaining sample (48 µl) in fractions 41-47, which showed

maximal BIAcore™ respons s, was analysed by SDS-PAGE on a 12% gel, followed by immunodetection of electroblotted proteins using an anti-STAT1 MAb. Proteins were electrophoretically transferred to PVDF (Immobilon, Millipore Waters. Cat. No. IPVH 000 10) membrane, and then immunodetection with STAT1 antibody was performed under conditions specified by the manufacturer, using detection by enhanced chemiluminescence (Amersham Cat. No. RPN 2109). The results are shown in Figure 3B.

The results show that treatment of the cells with IFN-γ causes both a net increase in IFN-γ R [$Y440_p$] binding (RU) and a shift of the binding factor to an apparently higher M_r, as determined by size exclusion-HPLC. This change correlates with an increased quantity and apparent M_r of immunoreactive STAT1 under non-denaturing conditions during size exclusion HPLC, suggesting its identity with the binding factor.

Example 4 Decrease of IFN-γ R [$Y440_p$] reactivity in STAT1-depleted HeLa nuclear extracts

HeLa cells were cultured as described in Example 2, but activated with IFN-γ only 15 minutes prior to extraction. Nuclear extracts were prepared as described by Dignam *et al* (1983), with the following modifications. The insoluble material removed after homogenization was resuspended in High-salt Buffer [20 mM Hepes pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT], and allowed to incubate for 30 minutes on ice. Nuclear matrices were removed by centrifugation for 20 minutes at 25,000 g. Nuclear extract and the anti-STAT1 affinity eluate were exchanged into assay buffer, as described in Example 2. The dilution of the extract prior to affinity absorption was chosen to yield a BIAcore™ response within the same range as the other sampl s.

The concentrated nuclear extract of HeLa cells, stimulated for 15 minutes with IFN-γ (IFN-γ activated

cells) prior to cell lysis and fractionation, was absorbed on to an anti-STAT1 MAb affinity resin (Transduction Laboratories. Following a washing cycle with assay buffer the affinity column was eluted with a buffer containing 3 M $MgCl_2$, 0.075 M HEPES pH 6.5, 25% ethyleneglycol. The relative BIAcore™ responses to immobilised IFN-γ R [$Y440_p$] peptide in dilutions of the extract before and after the affinity extraction, and of the column eluate, were determined. The remainder of the samples used for BIAcore™ analysis was analysed on a 12% SDS-PAGE gel followed by immunodetection of electroblotted proteins using an anti-STAT1 MAb as described in Example 3. In addition, an aliquot of the anti-STAT1 affinity matrix after $MgCl_2$-elution was analysed on the same gel. The results are shown in Figure 4. Panel A summarizes the BIAcore responses; Panel B shows the immunoblot results.

Example 5 Immunodetection of STAT1α-binding to an IFN-γ R [$Y440_p$] derivatised sensor chip

The nuclear extract of IFN-γ activated HeLa cells was purified on an IFN-γ R [$Y440_p$] affinity resin and the phenylphosphate eluate of the affinity matrix was exchanged into the appropriate assay buffer, as described in Example 2. The eluate was injected either directly (A, B) or after incubation with anti-STAT1 MAb (C) over a sensor chip derivatised with IFN-γ R [$Y440_p$]. In a second injection immediately following the first sample plug and prior to the start of the dissociation phase, either buffer (A, C) or anti-STAT1 MAb (B) was injected on to the same channel of the sensor chip. Figure 5 illustrates an overlay of BIAcore™ responses normalised to the same baseline. The individual injections are marked with an arrow labelled "1" (first injection) and "2" (second injection). The response levels at the start of the second injection are indicated.

Activated HeLa cells were fractionated as described in Example 4, and a 4 ml sample of the nuclear

fraction was extract d on 0.5 ml IFN-γ R [$Y440_p$]-agarose for 30 minutes at 0°C as described in Example 2. The affinity resin was washed quickly with 8 column volumes of assay buffer and eluted with one column volume of phenylphosphate (20 mM), collected in a single 0.5 ml fraction. The breakthrough from this column was loaded again under identical conditions. This second binding step increased recovery from the lysate to 90%. Both eluates were pooled, and a 90 µl aliquot incubated for 30 minutes at room temperature with 10 µl of anti-STAT1 MAb (200 µg/ml) diluted into assay buffer, or with the same volume of assay buffer. Aliquots (35 µl) of these sampl s were passed over a IFN-γ R [$Y440_p$]-derivatised sensor chip at 5 µl/minute and immediately after each sample plug had left the flow cell were followed by a second 35 µl injection of either running buffer or anti-STAT1 MAb at 1 µg/ml.

Thus either preincubation of cellular extracts with anti-STAT1 MAb prior to BIAcore™ analysis or injection of MAb on to IFN-γ R [$Y440_p$] sensor chip-bound factor resulted in an increased BIAcore™ response. In contrast, direct injection of the MAb on to the IFN-γ R [$Y440_p$]-derivatised sensor chip yielded no signal (Example 1), thus indicating that the primary antibody-enhanced binding response is due to STAT1.

Example 6 Absence of INF-γ R [Y440] Peptide Binding Component in Crude Cell Extracts, Detected by BIAcore™ and Immunotechnology Correlates with the Absence of STAT1 Protein

Parental (2fTGH) and mutant (U2A, U3A, U4A) human fibroblasts [as described elsewhere (Pellegrini *et al*, 1989; McKendry *et al*, 1991)] were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (vol./vol.) heat-inactivat d foetal calf serum and 5 µM L-glutamine. The cells were grown to 80% confluency, and harvested, following 2 washes in ice cold PBS supplemented

with 0.1 mM Na_3VO_4, in 1 ml trypsin-versene. Following trypsinisation, cells were washed twic in DMEM plus 10% foetal calf serum, and twice in ice-cold PBS supplemented with 0.1 mM Na_3VO_4. HeLa S3 cells were grown as described in Example 2. Cytoplasmic extracts were then prepared as described in Example 2.

The presence of STAT1 in samples (48 µl) of 10-fold concentrated cytosolic extracts, prepared as described above, was analysed by SDS-PAGE on a 7.5% gel followed by immuno-detection of electroblotted proteins using an anti-STAT1 Mab (as described in Example 3). This is presented in Figure 6A, and shows that STAT1 is not present in line U3A.

BIAcore™ detection of IFN-γ R [$Y440_p$] binding activity in the above samples was carried out as follows. Samples of 10-fold concentrated cytosolic extracts, prepared as described above, were incubated either with IFN-γ R [$Y440_p$] affi agarose or IFN-γ R [Y440] affi agarose, aspirated after 30 minutes at room temperature, and then injected at 5 µl/minutes onto BIAcore™ sensor chips, derivatised on parallel channels with IFN-γ R [Y440] or IFN-γ R [$Y440_p$] as described in Example 1.

BIAcore™ detection of IFN-γ R [$Y440_p$] binding activity in samples of 10-fold concentrated cytosolic extracts, prepared as described above, was carried out on sensor chips, derivatised on parallel channels with IFN-γ R [Y440] or IFN-γ R [$Y440_p$] as described in Example 1. The response sensorgrams of samples aspirated after 30 minutes incubation at room temperature with IFN-γ R [$Y440_p$] affi agarose, subtracted (BIAevaluation software ver.2.1) from the response in samples incubated with IFN-γ R [Y440] affi agarose are indicated in Figure 6B. No response to any of the extracts was detected on the IFN-γ R [Y440] derivatised channel.

Identical experiments wer carried out substituting the IFN-γ R [Y440] peptide with a phosphopeptide (IFN-α R [$Y466_p$]) corresponding to residues

460-474 of the IFN-α R (FLRCINYVFFPSLKP; SEQ. ID. NO. 2) and synthesised as described in Example 1. The relative BIAcore™ responses following injection of each cell extract were determined after 575 seconds (indicated by the arrow in Figure 6B). Figure 6C compares the responses of each cell extract to both phosphopeptides, plotted in relative response units.

Parallel aliquots of the peptide affi-agarose treated samples analysed in Figure 6B were examined by SDS-PAGE on a 7.5% gel followed by immunodetection of electroblotted proteins using an anti-STAT1 MAb as described in Example 3. The results, shown in Figure 6D, confirm that the approach taken above is valid, in that treatment of these extracts with phosphopeptide affi-agarose effectively depletes STAT1 protein. Furthermore, the absence of STAT1 in U3A extracts correlates with the lack of BIAcore™ response depicted in the sensorgram of Figure 6B.

Example 7 Other Potential STAT Binding Sites

Tyrosine-containing amino acid sequences in which the tyrosine can be phosphorylated are present in a numb r of proteins which have been reported in the literature. Some of these phosphotyrosine residues are located upon cytokine receptors, whereas others are found towards the C-terminal end of the STATs themselves. The putative STAT binding sites are summarized in Table 1, with the location of the phosphotyrosine identified in brackets. This location uses sequence numbering as indicated by mutation analysis.

Table 1

Receptor (Species)	Sequence	Binds	Reference
EPO-R (Human)	AQDTYLVLDEWLL (Y367) (SEQ. ID. NO. 3)	(STAT5)	Damen *et al*, 1995
IL-4 Rα	PAGGYQEFVQAVK (Y575) (SEQ. ID. NO. 4)	(STAT6)	Hou *et al*, 1994
	GGPGYKAFSSLLSS (Y603) (SEQ. ID. NO .5)	(STAT6)	Harpur, 1995
PRL (Human)	GGLDYLDPACFT (Y580) (SEQ. ID. NO. 6)	(STAT5)	Lebrun *et al*, 1995
gp130 (Human)	VHSGYRHQVPSVQV (Y765) (SEQ. ID. NO. 7)	(STAT3)	Stahl *et al*, 1995
LIF-R (Human)	VQSMYQPQAKPEE (Y981) (SEQ. ID. NO. 8)	(STAT3)	Stahl *et al*, 1995
	GGAGYKPQMHL (Y1001) (SEQ. ID. NO. 9)	(STAT3)	Harpur, 1995
G-CSF-R (Murine)	LVQAYVLQGPD (Y728) (SEQ. ID. NO. 10)	(STAT3)	Harpur, 1995
STAT1α (Human)	KGTGYIKTELISV (Y701) (SEQ. ID. NO. 11)		Shuai *et al*, 1993

Table 1
(continued)

Receptor (Species)	Sequence	Binds	Reference
STAT2 (Human)	YLKHRLIV (Y690) (SEQ. ID. NO. 12)		Shuai *et al*, 1993
STAT3 (Murine)	SAAPYLKTKFIC (Y705) (SEQ. ID. NO. 13)		Predicted in Zhong *et al*, 1994
STAT4 (Murine)	GDKGYVPSVFIP (Y694) (SEQ. ID. NO. 14)		Predicted in Zhong *et al*, 1994
STAT5 (Ovine)	AVDGYVKPQIK (Y694) (SEQ. ID. NO. 15)		Gouilleux *et al*, 1994
STAT6/IL-4 STAT (Human)	DGRGYVPATIK (Y641) (SEQ. ID. NO. 16)		Predicted in Hou *et al*, 1994

EPO : erythropoietin

PRL : prolactin

LIF : leukaemia inhibitory factor

Only the STAT1α Y701 is demonstrably a STAT binding site, while the other sites have been shown to be strong candidates for STAT binding by virtue of the fact that either phenylalanine substitution of the tyrosine results in loss of STAT binding (for example in the case of the EPO-R Y343 or the PRL-R Y580), or the sites lie in a region which appears to be required for signal transduction down-stream of a particular cytokine receptor (for example in the case of the LIF-R and gp130). A further class of predicted sites has been derived on the basis of what is known about STAT binding sites, coupled with what is known in the field of cytokine receptor-mediated signal transduction (usually denoted as Harpur, 1995).

The use of specific components of the IFN-γ signalling cascade and synthetic analogues thereof in an BIAcore™ biosensor-based assay to study initial steps of this pathway in detail has been described herein. As a minimal, functionally critical portion of the α-chain of the cytokine receptor, a peptide sequence surrounding a tyrosine residue (Tyr_{440}) which in its phosphorylated form is essential for biological responsiveness (Farrar *et al*, 1991, Greenlund *et al*, 1994) was immobilized onto the sensor chip of the biosensor. The efficacy of the coupling reaction and the integrity and accessibility of the phosphorylated tyrosine was demonstrated by monitoring, in real time, the binding of an anti-phosphotyrosine MAb to the peptide (Example 1). HeLa cell extracts and fibroblast extracts (2fTGH, U2A and U4A) were shown to contain a component that bound specifically to the sensor chip-immobilized, phosphorylated form of the receptor peptide.

Specificity was demonstrated by:

i) competition with free IFN-γ R [$Y440_p$] p ptide

ii) lack of response to IFN-γ R [Y440] peptide

iii) depletion of the IFN-γ R [$Y440_p$] binding

factor from the crude xtract with immobilized receptor peptide (Example 2).

iv) failure of extracts of U3A mutant cells, which lack STAT1, to generate a binding response to IFN-γ [$Y440_p$] peptide (Example 6).

Treatment of the cells with IFN-γ causes both a net increase in IFN-γ R [$Y440_p$] binding (RU) and a shift of the binding factor to an apparently higher M_r as determined by size exclusion-HPLC. This change correlates with an increased quantity and apparent M_r of immunoreactive STAT1, suggesting its identity with the binding factor (Example 3).

Three approaches were taken to verify this identity. First, immunodepletion of cellular extract with an anti-STAT1 affinity resin resulted in a titratable net reduction in IFN-γ R [$Y440_p$] binding (RU). The successful retention of STAT1 on the affinity resin was confirmed by western analysis (Example 4; Figure 4 lane 4), which also revealed "bleeding" of MAb together with antigen from the affinity resin (lanes 2, 5-7). Elution of the affinity column with 3M $MgCl_2$ yielded a small but titrable BIAcore™ response, suggesting unusually high avidity of the anti-STAT1 MAb.

Second, either preincubation of cellular extracts with anti-STAT1 MAb prior to BIAcore™ analysis or injection of MAb on to IFN-γ R [$Y440_p$] sensor chip-bound factor resulted in an increased BIAcore™ response. In contrast, injection of the MAb on to the IFN-γ R [$Y440_p$]-derivatised sensor chip gave no signal.

Finally, application of cellular extracts from the mutant fibroblast cell line U3A, which completely lacks STAT1α or STAT1β proteins, failed to generate a binding response to the IFN-γ R [$Y440_p$] peptide. Thus it is clear that the presence of STAT1 is required for the binding

response detected towards the IFN-γ R [$Y440_p$] peptide.

Taken together, these findings demonstrate that the local sequence of relatively short peptides contains sufficient information to enable their use as physiologically relevant affinity reagents. Thus a wide range of biomolecular interactions can be analysed using the approach presented herein.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

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(2) INFORMATION FOR SEQ ID NO. 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1

Lys Ala Pro Thr Ser Phe Gly Tyr Asp Lys Pro His Val Leu Val Asp
5 10 15

(2) INFORMATION FOR SEQ ID NO. 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2

Phe Leu Arg Cys Ile Asn Tyr Val Phe Phe Pro Ser Leu Lys Pro
5 10 15

(2) INFORMATION FOR SEQ ID NO. 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3

Ala Gln Asp Thr Tyr Leu Val Leu Asp Glu Trp Leu Leu
5 10

(2) INFORMATION FOR SEQ ID NO. 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4

Pro Ala Gly Gly Tyr Gln Glu Phe Val Gln Ala Val Lys
5 10

(2) INFORMATION FOR SEQ ID NO. 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5

Gly Gly Pro Gly Try Lys Ala Phe Ser Ser Leu Leu Ser Ser
5 10

(2) INFORMATION FOR SEQ ID NO. 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6

Gly Gly Leu Asp Tyr Leu Asp Pro Ala Cys Phe Thr
5 10

(2) INFORMATION FOR SEQ ID NO. 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7

Val His Ser Gly Tyr Arg His Gln Val Pro Ser Val Gln Val
5 10

(2) INFORMATION FOR SEQ ID NO. 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8

Val Gln Ser Met Tyr Gln Pro Gln Ala Lys Pro Glu Glu
5 10

(2) INFORMATION FOR SEQ ID NO. 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9

Gly Gly Ala Gly Tyr Lys Pro Gln Met His Leu
5 10

(2) INFORMATION FOR SEQ ID NO. 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10

Leu Val Gln Ala Tyr Val Leu Gln Gly Pro Asp
5 10

(2) INFORMATION FOR SEQ ID NO. 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11

Lys Gly Thr Gly Tyr Ile Lys Thr Glu Leu Ile Ser Val
5 10

(2) INFORMATION FOR SEQ ID NO. 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12

Tyr Leu Lys His Arg Leu Ile Val
5

(2) INFORMATION FOR SEQ ID NO. 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13

Ser Ala Ala Pro Tyr Leu Lys Thr Lys Phe Ile Cys
5 10

(2) INFORMATION FOR SEQ ID NO. 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14

Gly Asp Lys Gly Tyr Val Pro Ser Val Phe Ile Pro
5 10

(2) INFORMATION FOR SEQ ID NO. 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15

Ala Val Asp Gly Tyr Val Lys Pro Gln Ile Lys
5 10

(2) INFORMATION FOR SEQ ID NO. 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16

Asp Gly Arg Gly Tyr Val Pro Ala Thr Ile Lys
5 10

CLAIMS:

1. A receptor peptide having a sequ nce corresponding to a tyrosine-containing region an intracellular domain of a cytokine, a hormone receptor or a JAK protein tyrosine kinase, wherein said peptide comprises a tyrosine residue capable of being phosphorylated, wherein said peptide, in its phosphorylated form, is able to bind a cytoplasmic transcription factor of the STAT family.

2. The receptor peptide of Claim 1, wherein said peptide is derived from a receptor for a protein selected from the group consisting of growth hormone, prolactin, leukaemia inhibitory factor, erythropoietin, ciliary neurotrophic factor, interferon-γ, interferon-α, interferon-β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF, CSF-1, erythropoietin, EGF, PDGF and Oncostatin M.

3. The receptor peptide of Claim 2, wherein said peptide is derived from a receptor for a protein selected from the group consisting of growth hormone, prolactin, interferon-γ, interferon-α, interferon-β, IL-2, IL-4, IL-5, GM-CSF and erythropoietin.

4. The receptor peptide of Claim 1, wherein said peptide has the sequence KAPTSFGYDKPHVLVD, corresponding to residues Lys_{433} to Asp_{448} of the amino acid sequence of the interferon-γ receptor protein, or a homologue or variant thereof, and wherein said peptide, in its phosphorylated form, has th ability to bind STAT1α.

5. The receptor peptide of Claim 1, wherein said peptide has the sequence FLRCINYVFFPSLKP, corresponding to residues Phe_{460} to Pro_{474} of the intereferon-α receptor protein, or a homologue or variant thereof, and wherein said peptide, in its phosphorylated form, has the ability to bind STAT2.

6. A method of screening a molecule for its ability to interact with a receptor peptide according to Claim 1, comprising exposing a receptor peptide of Claim 1 to a molecule to be screened, and measuring the degree of interaction between said receptor peptide and said molecule.

7. The method of Claim 6, wherein said receptor peptide is a receptor for a protein selected from the group consisting of growth hormone, prolactin, leukaemia inhibitory factor, erythropoietin, ciliary neurotrophic factor, interferon-γ, interferon-α, interferon-β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF, CSF-1, erythropoietin, EGF, PDGF and Oncostatin M.

8. The method of Claim 7, wherein said receptor peptide is a receptor for a protein selected from the group consisting of growth hormone, prolactin, interferon-γ, interferon-α, interferon-β, IL-4, IL-5, GM-CSF and erythropoietin.

9. The method of Claim 6, wherein said receptor peptide has the sequence KAPTSFGYDKPHVLVD, corresponding to residues Lys_{433} to Asp_{448} of the amino acid sequence of the interferon-γ receptor protein, or a homologue or variant thereof, wherein said peptide, in its phosphorylated form, has the ability to bind STAT1α.

10. The method of Claim 6, wherein said receptor peptide has the sequence FLRCINYVFFPSLKP, corresponding to residues Phe_{460} to Pro_{474} of the interferon-α receptor protein, or a homologue or variant thereof, wherein said peptide, in its phosphorylated form, has the ability to bind STAT2.

11. A method of measuring the ability of a molecule to inhibit or promote interaction between a receptor peptide of Claim 1, and a molecule known to be able to bind to said receptor, comprising exposing a receptor peptide of Claim 1 to a

known molecule having th ability to bind to said receptor in the presence of a putative inhibitor or promoter, and measuring the ability of the putative inhibitor to inhibit said binding.

12. The method of Claim 11, wherein said receptor is a receptor for a protein selected from the group consisting of growth hormone, prolactin, leukaemia inhibitory factor, erythropoietin, ciliary neurotrophic factor, interferon-γ, interferon-α, interferon-β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF, CSF-1, erythropoietin, EGF, PDGF and Oncostatin M.

13. The method of Claim 12, wherein said receptor is a receptor for a protein selected from the group consisting of growth hormone, prolactin, interferon-γ, interferon-α, interferon-β, IL-4, IL-5, GM-CSF and erythropoietin.

14. The method of Claims 10, wherein the receptor peptide has the sequence KAPTSFGYDKPHVLVD, corresponding to residues Lys_{433} to Asp_{448} of the amino acid sequence of the interferon-γ receptor protein, or a homologue or variant thereof, wherein said peptide, in its phosphorylated form, has the ability to bind STAT1α.

15. The method of Claim 11, wherein said receptor peptide has the sequence FLRCINYVFFPSLKP, corresponding to residues Phe_{460} to Pro_{474} of the intereferon-α receptor protein, or a homologue or variant thereof, wherein said peptide, in its phosphorylated form, has the ability to bind STAT2.

16. The method of Claim 6, wherein binding to the receptor peptide is measured by a method selected from the group consisting of radioactivity detection, fluorescence detection, immunoassay, affinity assay, fluorescence-activat d cell sorting, bioassay, and biosensor assay.

17. The method of Claim 11, wherein binding to the receptor peptide is measured by a method selected from the group consisting of radioactivity detection, fluorescence detection, immunoassay, affinity assay, fluorescence-activated cell sorting, bioassay, and biosensor assay.

FIG. 1

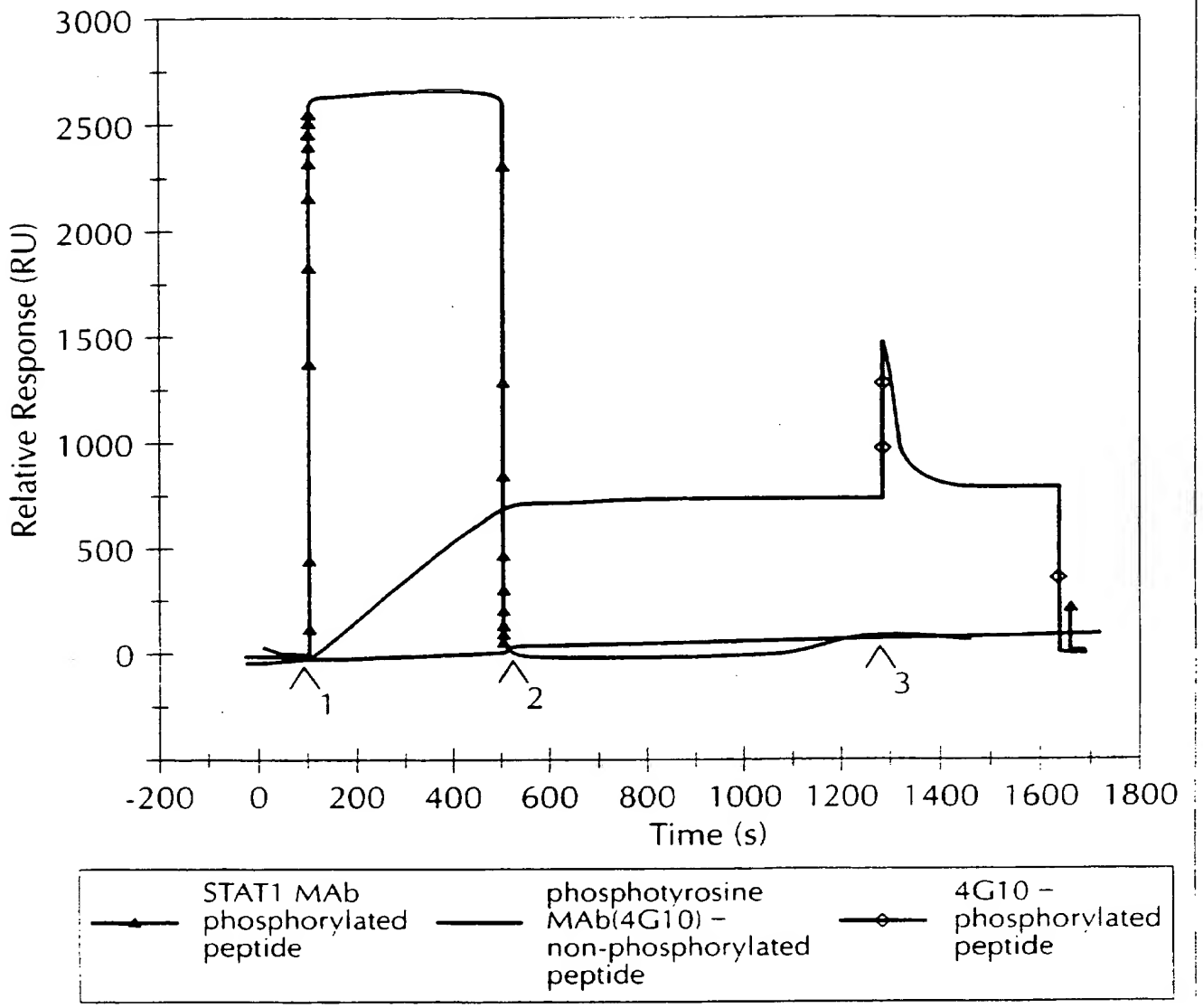


FIG. 2

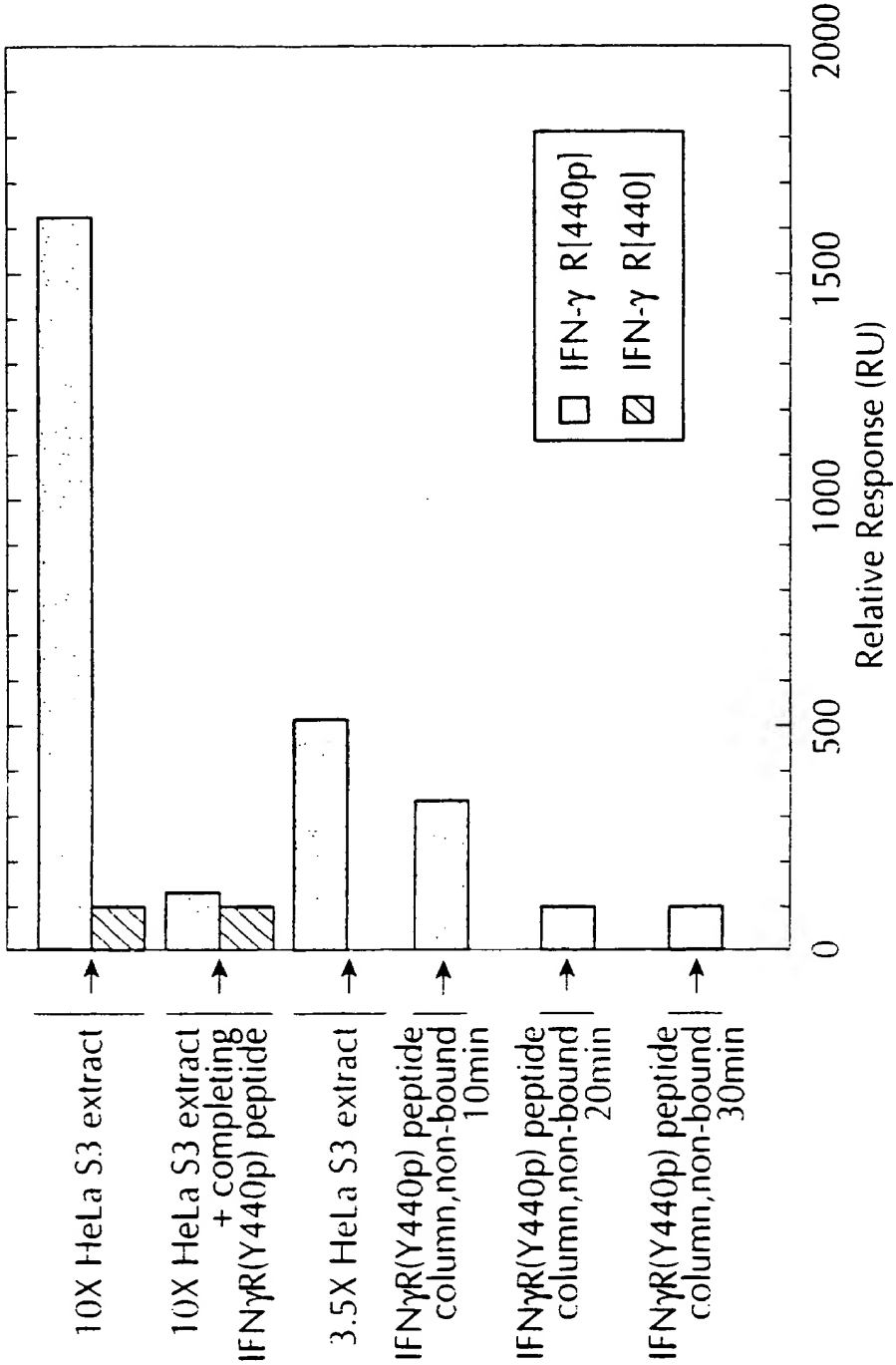


FIG. 3A

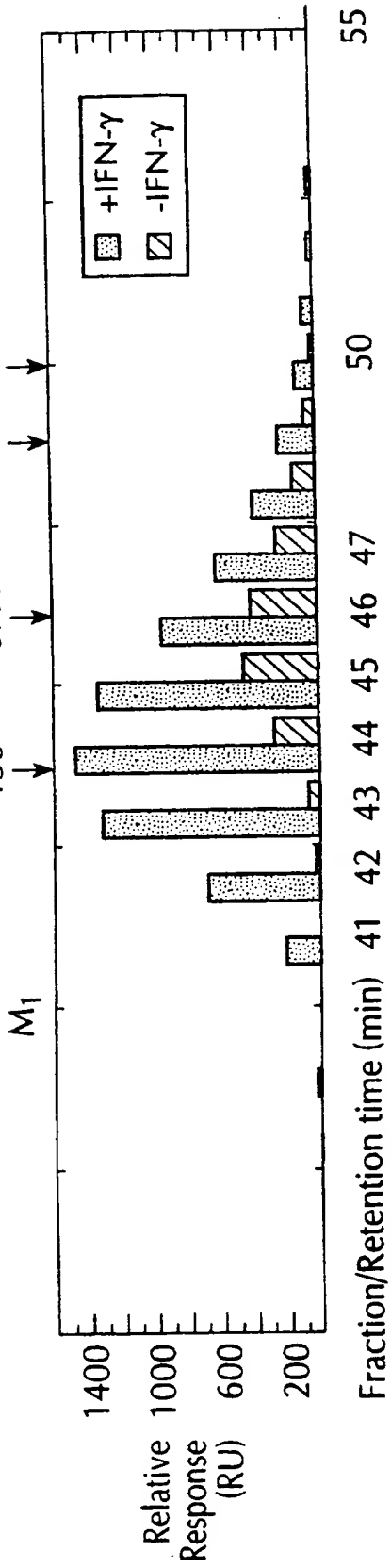


FIG. 3B

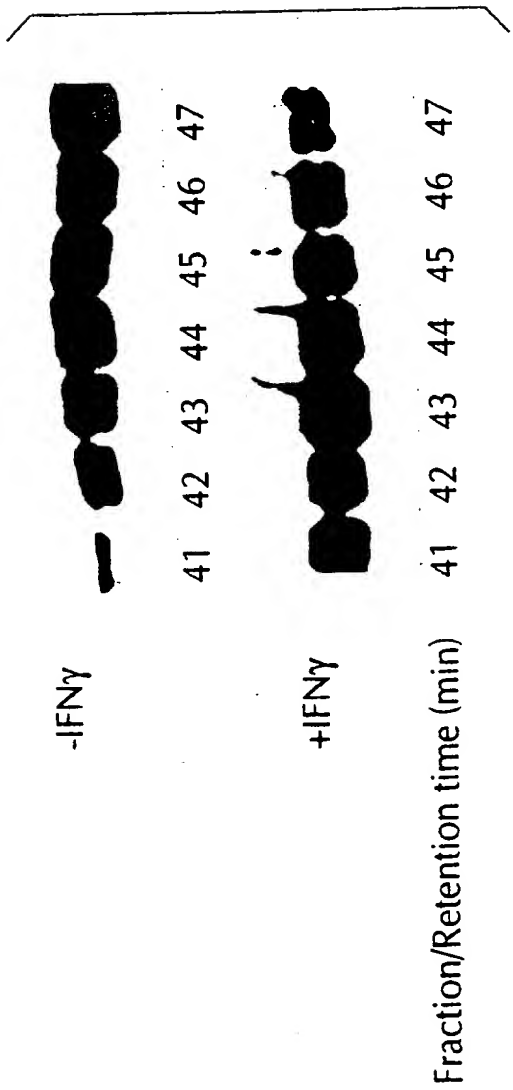


FIG. 4A

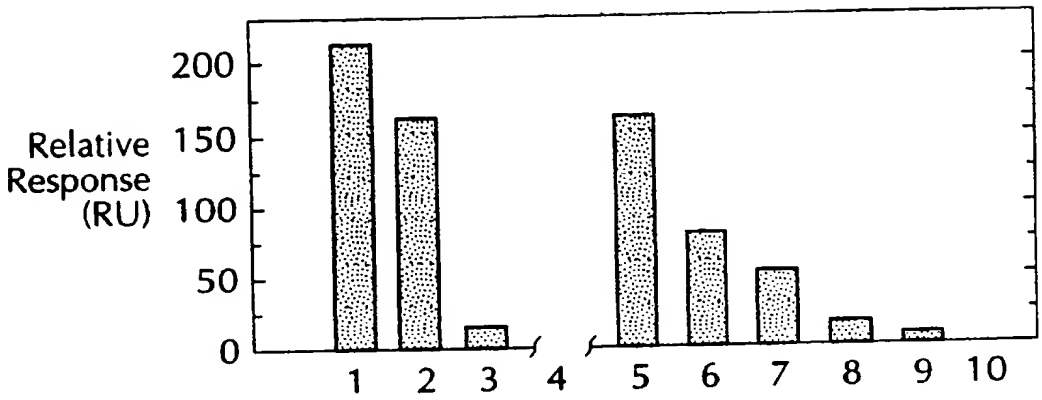


FIG. 4B

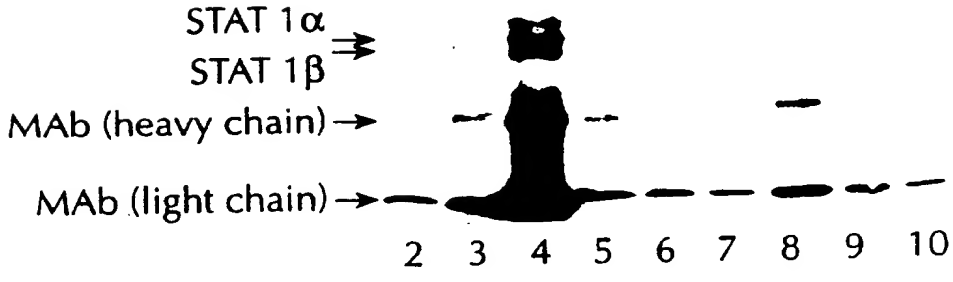
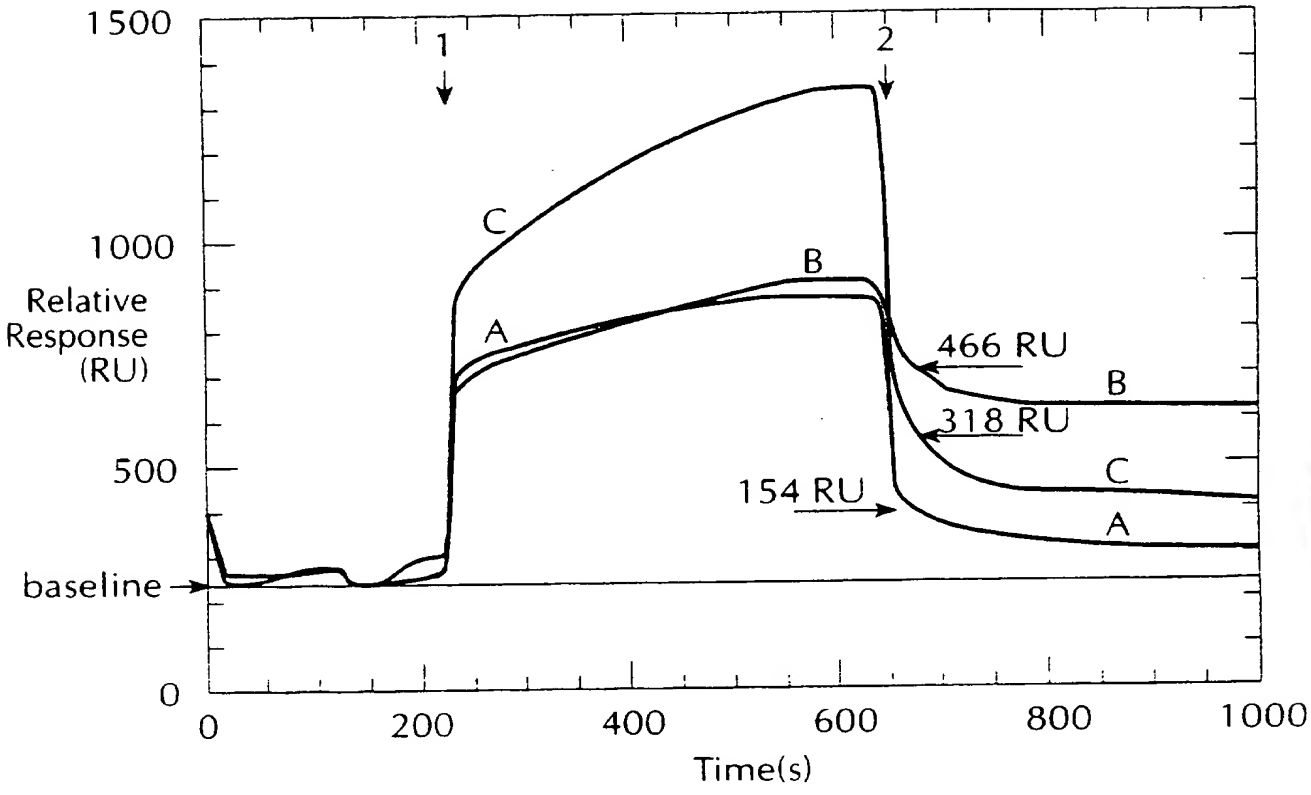


FIG. 5



Sample and injection point:

A	B	C
1: IFNγR[Y440$_p$] column eluate	1: IFNγR[Y440$_p$] column eluate	1: IFNγR[Y440$_p$] column eluate plus STAT1 MAb
2: buffer	2: STAT1 MAb	2: buffer

FIG. 6A

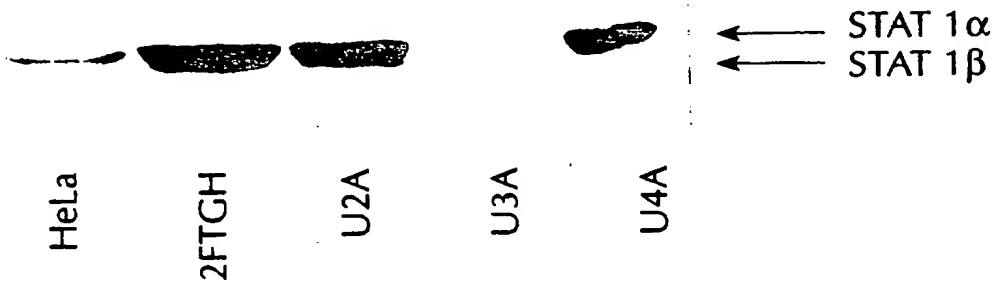


FIG. 6B

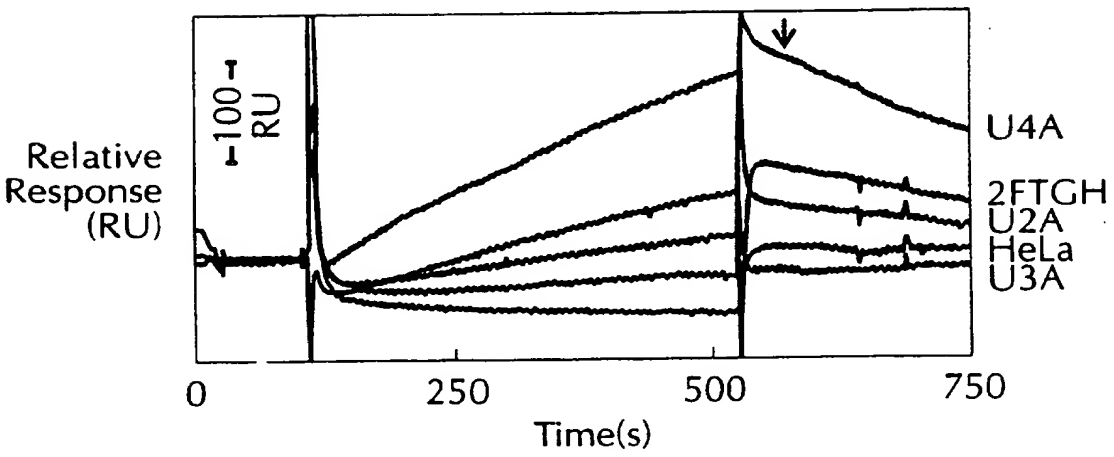


FIG 6C

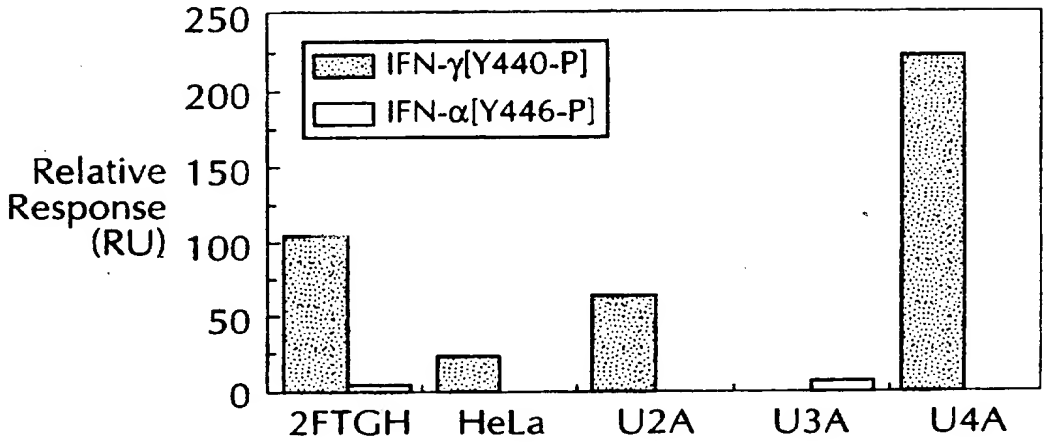
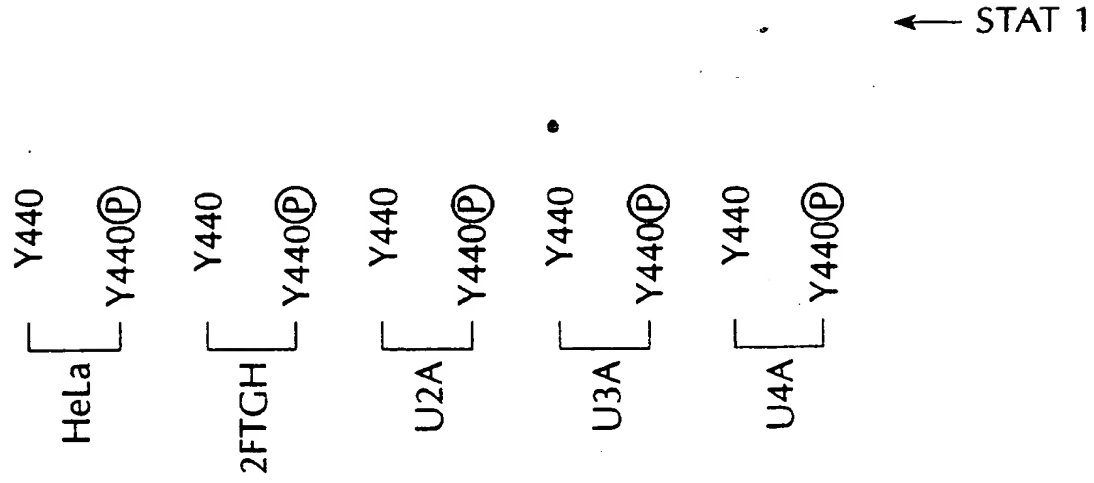


FIG. 6D



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/16988

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 1/00; G01N 33/53
US CL :530/300, 325; 435/7.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/300, 325; 435/7.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Greenlund, Ligand-induced IFN-gamma tyrosine kinase phosphorylation couples the receptor to it's signal transduction system. EMBO Journal, April 1994, Vol. 13, No. 7, especially pages 1592-1598.	1-5
X	Larner et al. Tyrosine Phosphorylation of DNA Binding Proteins by multiple Cytokines. SCIENCE. 24 September 1993, Vol. 261, especially pages 1731-1733.	1-5
X	Ruff-Jamison et al. Induction of EGF and Interferon-gamma of Tyrosine Phosphorylated DNA Binding Proteins in Mouse Liver Nuceli. SCIENCE. 24 September 1993, Vol. 261, especially pages 1734-1736.	1-5

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
22 APRIL 1996	03 MAY 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Kenneth A. Sorensen Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/16988

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Sadowski et al. A Common Nuclear Signal Transduction Pathway Activated by Growth Factor and Cytokine Receptors. SCIENCE. 24 September 1993, Vol. 261, especially pages 1739-1743.	1-5
X	Shuai et al. A single phosphotyrosine residue of Stat91 required for gene activation by interferon-gamma. Science. September 1993, Vol. 261, especially pages 1744-1746.	1-5
Y	Hou et al. An Interleukin-4-Induced Transcription Factor: IL-4 Stat. SCIENCE. 16 September 1994, Vol. 265, espcecially pages 1701-1705	1-5
Y	Dusanter-Fourt et al. Identification of JAK protein tyrosine kinases as signalling molecules for prolactin. Functional analysis of prolactin receptor and prolactin - erythropoietin receptor chimera expressed in lympoid cells. EMBO. Journal June 1994, Vol.13, No. 11, especially 2584-2590.	1-5
X	Auget et al. Molecular cloning and expression of the human interferon-gamma receptor. Cell. 1988, Vol. 55, No. 2, 21 October 1988, especially 274-80.	4, 9, 14
X	Uze, et al. Genetic Transfer of a Functional Human Interferon-alpha Receptor into Mouse Cells: Cloning and Expression of its cDNA. Cell. 1990, Vol. 60, No. 2, 26 January 1990, especially pages 226-234.	5,10,15
X, P ----- Y, P	US 5,463,023 A (SCHREIBER) 31 October 1995, Figure 5, columns 1-12.	6-8, 11-14 ---------- 16-17
X, P ------ Y, P	US 5,470,952 A (STAHL) 28 November 1995, Figure 1, columns 1-12.	6-8,11-14 ---------- 16-17

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/16988

Box I Observations where certain claims were found unsearchable (Continuation of item 1 f first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/16988

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, medline, embase, biosis, confsci, dissabs, caplus, jicst-eplus, wpids, patosep;
Search terms: intracellular domain, cytokine, hormone, jak, cytoplasmic transcription factor, bind, stat, stat1 gamma, stat family, stat1, p91, tyro440, biocore, interfereon gamma

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-10, 14, 16, drawn to a receptor peptide and a method of screening molecules for ability to interact with said receptor peptide.
Group II, claim(s) 11-13, 15,17, drawn to a method of measuring the ability of molecules to inhibit or promote interaction between a receptor peptide and a known molecule.
The inventions listed as Groups I & II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
Group I consists of claims drawn to a receptor peptide and a method of screening molecules for their ability to interact with said receptor peptide. The special technical feature is the ability of the peptide to bind a cytoplasmic transcription factor of the STAT family.
Group II consists of claims drawn to a method of measuring the ability of molecules to inhibit or promote interactions between a receptor peptide and a known molecule. The special technical feature differs from group one in that it involves a method usingthree compounds, the third compound is materially different from the compounds used in the peptide and process of group I.